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**Citation for published version:**

Markose, D, Kirkland, P, Ramachandran, P & Henderson, NC 2018, 'Immune cell regulation of liver regeneration and repair', *Journal of Immunology and Regenerative Medicine*.  
<https://doi.org/10.1016/j.regen.2018.03.003>

**Digital Object Identifier (DOI):**

[10.1016/j.regen.2018.03.003](https://doi.org/10.1016/j.regen.2018.03.003)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Version created as part of publication process; publisher's layout; not normally made publicly available

**Published In:**

Journal of Immunology and Regenerative Medicine

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# Accepted Manuscript

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PII: S2468-4988(18)30004-0

DOI: [10.1016/j.regen.2018.03.003](https://doi.org/10.1016/j.regen.2018.03.003)

Reference: REGEN 7

To appear in: *Journal of Immunology and Regenerative Medicine*

Revised Date: 19 March 2018

Accepted Date: 23 March 2018

Please cite this article as: Markose D, Kirkland P, Ramachandran P, Henderson NC, Immune cell regulation of liver regeneration and repair, *Journal of Immunology and Regenerative Medicine* (2018), doi: 10.1016/j.regen.2018.03.003.

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## Immune cell regulation of liver regeneration and repair

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## Abstract

The liver harbors a rich, diverse spectrum of innate and adaptive immune cells. In homeostasis, these immune cells perform host defense against gut-derived pathogens and mediate tolerance to self-antigens. Following tissue injury there are complex interactions within the immune cell compartment which regulate liver regeneration and repair. Partial hepatectomy (PHx) and acetaminophen induced liver injury (AILI) are clinically relevant models of liver injury, which are commonly used to study liver regeneration. Here we discuss how the innate and adaptive immune systems influence liver regeneration and repair following acute hepatic injury.

## Introduction

The mammalian liver is a multifunctional organ with a unique ability to regenerate following tissue injury. This evolutionary adaptation sets the liver apart from other organs, enabling it to detoxify various drugs and toxins whilst simultaneously regenerating after injury. Despite this well-orchestrated process of regeneration, mortality rates from liver disease have continued to rise inexorably since the 1970s<sup>1</sup>. In advanced chronic liver disease, the regenerative and reparative capacity of the liver diminishes and transplantation is the only curative option for patients<sup>2</sup>. Shortages in the availability of donor organs and the requirement for life-long immunosuppression following liver transplantation, means that potent pro-regenerative therapies are urgently required. A deeper understanding of the cellular and molecular mechanisms which drive liver repair and regeneration, will hopefully facilitate the development of potent new therapies for patients with liver disease<sup>3</sup>.

58

59 Around 80% of the liver mass is comprised of hepatocytes, and these cells are  
60 integral to the myriad functions of the liver, namely: metabolic homeostasis, storage  
61 of nutrients, secretion of bile and detoxification of harmful drugs. Hepatocytes are  
62 quiescent in homeostatic conditions, at any given time less than 1-2% of hepatocytes  
63 are in the cell cycle <sup>4</sup>, but during injury they re-enter the cell cycle. Although liver  
64 regeneration is characterized by proliferation of hepatocytes, the induction of  
65 proliferation is also achieved through cytokine-driven interactions with non-  
66 parenchymal cells (NPCs), which constitute approximately 20% of the liver mass.  
67 These cells include liver sinusoidal endothelial cells (LSEC), hepatic stellate cells  
68 (HSC), and hepatic immune cells (Figure1).

69

70 As the largest internal organ, exposed to both the portal and arterial circulation, the  
71 liver harbors an extensive reservoir of resident immune cells. These hepatic immune  
72 cells form a “firewall” to mediate host defense against blood-borne pathogens and  
73 maintain tolerance to non-self-antigens from nutrients or resident microbiota <sup>5</sup>.  
74 Following liver injury, dynamic changes in the hepatic immune cell compartment are  
75 observed at a tissue, cellular and genetic level, which have a critical role in  
76 orchestrating liver regeneration and repair.

77

## 78 **Models of Liver Regeneration**

79

80 In order to study the mechanisms underpinning liver regeneration, pre-clinical  
81 models have been utilized to induce hepatocyte injury, and stimulate parenchymal  
82 and non-parenchymal cell replication <sup>6,7</sup>. These rodent models are frequently used to

dissect hepatic immunity in homeostasis and disease and highlight their importance in the regulation of liver regeneration and repair. Advancements in transgenic animal technology, which enable selective interrogation of immune cell function, have greatly increased our understanding of the role of immune cells in liver injury and repair<sup>8</sup>. However, there are distinct differences in the immune cell composition and marker expression between humans and rodents, and therefore, as always, care must be taken in extrapolating data from rodents to humans. Despite these limitations, these models continue to give important mechanistic insights into how the immune cell compartment regulates liver regeneration.

### **Partial Hepatectomy (PHx)**

In a clinical setting, liver resection is one of the main treatments for hepatic malignancies and the repair of trauma<sup>9</sup>. To study the mechanisms underpinning this remarkable regenerative process, rodent partial hepatectomy (PHx) has become a standard pre-clinical model, where 2/3 of the rodent liver is removed surgically<sup>7,9</sup>. Once the lobules are removed, regeneration is initiated immediately, with the remnant lobes enlarging via compensatory hyperplasia, until the original mass of the liver is reached, a process which takes approximately 7 days<sup>7,10</sup>. These rodent models have facilitated a detailed interrogation of this regenerative process, highlighting cellular kinetics, molecular mechanisms and key signaling pathways<sup>10,11</sup>. Hepatocyte proliferation in C57BL/6 mice peaks around 36-48 hours post-resection<sup>12</sup>. This process is mediated by complex cross-talk between hepatocytes and NPCs<sup>11</sup>. Importantly, this model does not reflect the significant inflammatory

responses that can occur in pathological conditions of human acute and chronic liver injury, where liver regeneration may be compromised.

## **AILI**

Acute liver failure (ALF) in humans is associated with high mortality rates, brought about by hepatic parenchymal cell death, extensive intra-hepatic inflammation and a systemic inflammatory response (SIRS)<sup>13,14</sup>. This overwhelming inflammation contributes to multiple organ dysfunction and death<sup>15</sup>. Acetaminophen (APAP) poisoning is the commonest cause of ALF in western countries<sup>3</sup>. Following ingestion of recommended doses of APAP, hepatocytes are protected against its toxic metabolite: N-acetyl-p-benzoquinone (NAPQI), due to detoxification by glutathione. However, at higher doses of APAP, glutathione is depleted by NAPQI, and leads to hepatocyte death. In toxic injuries, liver regeneration is a dose dependent process, it increases with the extent of hepatic injury until a threshold is reached where increasing hepatic injury inhibits the regenerative process, accelerating the progression to ALF<sup>16</sup>. AILI can be modeled in rodents by a single dose of APAP, with similar histological and biochemical features to those seen in human ALF<sup>15,17</sup>.

APAP-induced liver injury (AILI) is a bi-phasic process, initial stages involve hepatocyte necrosis from covalent binding of NAPQI to cellular proteins causing mitochondrial dysfunction, oxidative stress and peroxynitrite formation<sup>18</sup>. The secondary phase of the injury is characterized by intrahepatic and systemic inflammation (Figure 2). Inflammation is triggered by the release of “danger associated molecular patterns” (DAMPs) such as high-mobility group box 1

(HMGB1) protein and heat shock protein-70 from damaged or dying hepatocytes, which then activate NPCs through surface-expressed toll-like receptors (TLRs)<sup>19,20</sup>. The use of animal models has enabled comprehensive analysis of the primary stages of AILI, which has increased our knowledge of the metabolism of APAP by hepatocytes, and the molecular mechanism by which necrosis occurs<sup>18,21</sup>. Despite this, the current initial therapeutic options for APAP-induced liver injury (AILI) are limited to the antidote: *N*-acetyl-cysteine (NAC). NAC reverses hepatotoxicity by quenching NAPQI, targeting the initial stages of the disease, so therefore must be given early after APAP poisoning to be effective<sup>22</sup>. Recent studies have demonstrated that the secondary immune response following hepatocyte death is a crucial determinant in disease progression and contributes to the evolution of extra-hepatic features like SIRS and multi-organ dysfunction<sup>13,15,23</sup>.

## **Immune Regulation of Liver Regeneration**

As the immune system plays a major regulatory role in the hepatic regenerative and reparative response, in this review we will describe the known functional roles of various cell lineages within the immune cell compartment, with emphasis on how these cells determine the outcome of hepatic injury and repair.

## **Macrophages/Monocytes**

When initially discovered, macrophages were described as phagocytic cells in the circulation, responsible for recognizing, engulfing and degrading foreign pathogens and cellular debris. Since then, in addition to bone marrow-derived circulating



monocytes/macrophages, distinct macrophage populations have been discovered in many tissues of the body<sup>24,25</sup>. Liver resident macrophages, termed Kupffer cells (KCs) represent approximately 20% of the hepatic NPC population<sup>26</sup>. They are now thought to have an embryonic origin in homeostasis, having the capacity to self-renew independently with no contribution from circulation-derived progenitors<sup>27</sup>. KCs have a role in immune surveillance and maintaining tolerogenic responses in the liver<sup>5,28</sup> (Figure 1). They capture and phagocytose blood borne food antigens and bacteria from the intestine, whilst preventing immune responses to harmless gut-derived antigens<sup>5,29,30</sup>. When homeostasis is disrupted by tissue injury, macrophages are critical mediators of tissue repair, from initiation and propagation to resolution of tissue injury<sup>22</sup>. KCs are the first immune cells to become activated through recognition of DAMPs via TLRs on their surface<sup>31,32</sup>. This results in the release of pro- and anti-inflammatory mediators and recruitment of circulatory-derived immune cells into the liver<sup>33</sup> (Figure 2A).

Resident and recruited macrophages seems to be critical for effective liver regeneration following PHx. Pre-treatment of mice with either liposomal clodronate or gadolinium chloride to deplete or inhibit macrophages respectively, resulted in impaired liver regeneration following PHx<sup>34</sup>. Liver regeneration is also impaired in mice where recruitment of monocytes is prevented following PHx<sup>35,36</sup>. The presumed mechanism is that macrophages release pro-reparative cytokines such as interleukin-6 (IL-6), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), and hepatocyte growth factor (HGF), to drive hepatocyte proliferation<sup>34</sup>. However, a detailed characterization of macrophage phenotype following PHx remains lacking. Furthermore, elevated colony stimulating factor 1 (CSF1), a mitogen and survival factor for macrophages is

seen in the serum of patients who have undergone PHx<sup>37</sup>, suggesting a role for macrophages in human liver regeneration.

In AILI the precise role of macrophages remains controversial, with contradictory studies reporting both deleterious and hepatoprotective functions. Using flow-cytometry-based phenotyping, three subsets of macrophages with distinct gene expression profiles have been shown to populate the murine liver following AILI: KCs, Ly6C<sup>Hi</sup> monocyte-derived macrophages (MoMFs) and Ly6C<sup>Lo</sup> MoMFs<sup>38,39</sup>. There are dynamic changes in these subsets of monocytes/macrophages during AILI<sup>38,40</sup>. KCs around the necrotic area are depleted during the necroinflammatory phase of AILI, whilst during the repair phase their numbers are re-established via self-renewal, independent of MoMFs<sup>38,41</sup>. Since these studies do not use a specific, discriminatory marker it is difficult to know whether “KC disappearance” signifies the death, migration or plasticity following AILI<sup>38,39,41</sup>. A recent study demonstrated how bacteria-induced early necroptosis of KCs is necessary to orchestrate polarization of macrophages to a pro-reparative phenotype, promoting liver repair following infection<sup>42</sup>. Future studies using a KC-specific fate mapping strategy could help elucidate whether “KC disappearance” has any similar functional relevance in AILI.

The role of KCs in AILI has been studied primarily using clodronate liposome mediated depletion. However, in addition to depleting KCs, clodronate liposomes also deplete other types of liver macrophages and monocytes. Therefore this is an imperfect experimental system for specifically studying the role of KCs in AILI. Despite these technical limitations, mice treated with clodronate liposomes display significantly increased hepatic injury and necrosis following AILI<sup>43–45</sup>. Cytokine

analysis of KCs during ALI demonstrates that these cells are major producers of hepatocyte mitogens such as IL-6, TNF- $\alpha$  and IL-10<sup>44,46</sup>. Interestingly, microarray profiling of KCs from a post-APAP livers compared to healthy livers display very little differences in their transcriptome<sup>38</sup>. More targeted, high resolution techniques, which also considers the spatial distribution of KCs could give significantly more information regarding their precise role in ALI.

Circulatory-derived monocytes and macrophages (MoMFs), that are distinct from resident KCs, have also been implicated in the pathogenesis of ALI<sup>38,39,47</sup>. During the necroinflammatory phase, Ly6C<sup>Hi</sup> monocytes can infiltrate the liver through C-C chemokine receptor 2 (CCR2) and C-C chemokine ligand 2 (CCL2)-dependent transmigration<sup>48,49</sup>. Once in the liver they differentiate into macrophages (MoMFs) and aggregate around the necrotic areas<sup>38,39</sup> (Figure 2A). Eliminating monocytes and MoMFs from the liver during ALI, by inhibition of CCR2 -CCL2 interaction, results in exaggerated hepatic injury<sup>38,50-52</sup>. Conversely, Ly6C<sup>Hi</sup> monocytes have an overall pro-inflammatory transcription profile, which can aggravate the early phase of ALI<sup>39</sup>. These apparently contradictory findings can be explained by the plasticity of MoMFs. Upon maturation, these pro-inflammatory Ly6C<sup>Hi</sup> monocytes acquire a pro-reparative phenotype, where angiogenic and tissue remodeling factors such as vascular growth factor A (VEGFA) and fibronectin are upregulated in these cells<sup>38</sup>. They polarize to ephemeral Ly6C<sup>Lo</sup> CCR2<sup>Lo</sup> CX3CR1<sup>Hi</sup> macrophages (MoMFs) that promote angiogenesis, tissue remodeling and necrotic cell clearance<sup>38</sup> (Figure 2B).

These data suggest that specific subsets of macrophages promote a niche which supports clearance of cellular debris and an anti-inflammatory phenotype, promoting

live repair and regeneration<sup>40,53</sup>. In addition to these pro-reparative responses macrophages also have a pivotal role in mediating the broader immune response during AILI. Due to increased gut permeability, the capacity of the liver to maintain an effective immune barrier against gut-derived pathogens is markedly reduced during liver injury<sup>54</sup>. Thus, activation of hepatic macrophages is a key regulatory process during liver injury, not only mediating tissue repair but also combating the influx of pathogens into the liver and preventing their spread systemically<sup>55</sup>. In line with this, sepsis and/or SIRS have been identified as major factors contributing to worsening hepatic encephalopathy in ALF, conferring poor prognosis<sup>13,15</sup>.

Macrophages have been shown to be important in human AILI. Pro-inflammatory and pro-resolution monocyte/macrophages, analogous to subsets identified in mouse models of AILI, have also been described in humans<sup>40,56</sup>. Recent studies in both humans and mice highlight secretory leukocyte protease inhibitor (SLPI)-reprogramming of macrophages to a pro-resolution phenotype (Figure 2B). This subset of macrophages express a tyrosine kinase receptor (MerTK), which promotes resolution of inflammation following AILI via enhanced phagocytic capabilities and neutrophil apoptosis<sup>40</sup>. Monocyte reduction and dysfunction can lead to a poor prognosis in AILI patients and promote ALF<sup>37,56</sup>. Consequently, macrophage-based therapies have been suggested as a treatment for ALF. This has been demonstrated as a proof of concept in mice, whereby administration of CSF1 was shown to promote liver regeneration by promoting KC and MoMF proliferation<sup>37</sup>. However, macrophages in the context of AILI can be highly dynamic, adapting rapidly to changes in the microenvironment<sup>57,58</sup>. Therefore, to therapeutically enhance the reparative capabilities of macrophages the signaling cues which promote

macrophage plasticity and how these changes temporally during AILI needs to be deciphered.

### **Dendritic Cell (DC)**

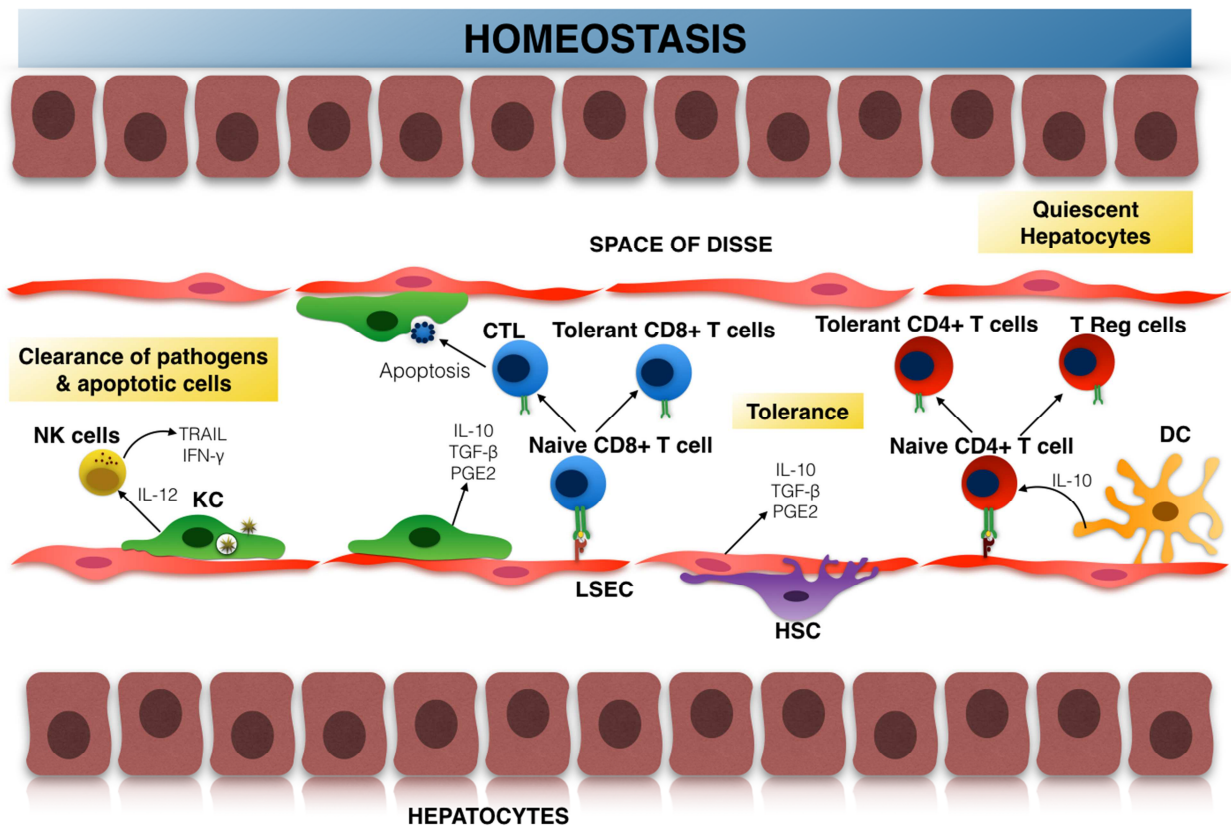
DCs are major antigen presenting cells (APC) in the liver, and constitute approximately 1% of hepatic NPCs<sup>59</sup>. They are primarily found in the portal region, with sparse distribution in the parenchyma<sup>60</sup>. In homeostasis, DCs capture and process antigens and migrate to lymphoid tissues to mediate tolerogenic responses, by interacting with effector lymphocytes<sup>59,61</sup> (Figure 1). Mitogens such as fms-like tyrosine kinase 3 ligand (Flt3L) and granulocyte macrophage colony-stimulating factor (GM-CSF), can be used to augment DC numbers in the liver<sup>62</sup>. Similar to macrophages, DCs represent a heterogeneous population of cells, consisting of multiple distinct subsets with varying functional capabilities<sup>63</sup>. Current classification of hepatic DCs in rodents have arisen from multiparametric flow cytometry experiments and visualization with immunohistochemistry and intravital imaging<sup>64–66</sup>. All DCs express MHCII and CD45 on their surface and based on additional markers, DCs have been broadly divided into two main subtypes: conventional DCs (cDCs) and plasmacytoid DCs (pDCs)<sup>63</sup>.

DCs have been shown to exhibit regeneration and repair properties and regulate liver regeneration<sup>67</sup>. PHx causes a marked increase in CD11c+ DC numbers, without any apparent surface phenotypic changes. In vivo expansion of DCs via administration of Flt3L significantly increases in liver regeneration<sup>67</sup>. DCs also display a pro-repair cytokine pattern through the upregulation of IL-10, an anti-inflammatory cytokine<sup>52</sup>, and downregulation of IFN- $\gamma$  mRNA, a pro-inflammatory

cytokine<sup>67,68</sup>. DCs have also been shown to influence other cell types such as T cells following PHx, promoting an anti-inflammatory milieu<sup>67</sup>.

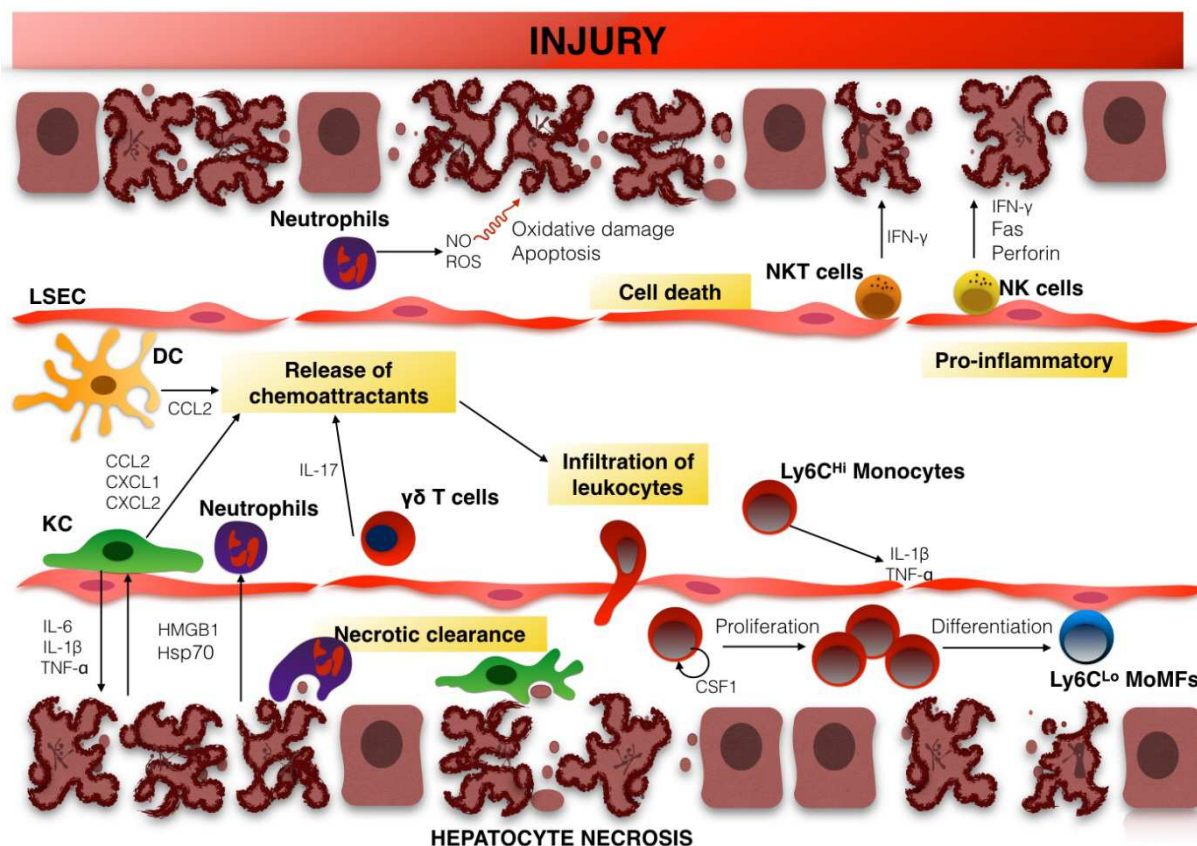
In AILI, DCs display a protective role. Transient depletion of DCs in CD11c.DTR mice results in increased hepatocyte necrosis with elevation in serum liver enzymes and increased mortality<sup>69</sup>. The precise mechanism by which they suppress injury and promote repair in this setting has not been fully elucidated. APAP overdose does not result in DC expansion but changes in the DC immune-phenotype, with increased production of IL-6, TNF- $\alpha$  and CCL2<sup>69,70</sup> (Figure 2). They have also been shown to negatively influence pro-inflammatory cell types such as neutrophils and natural killer cells<sup>69</sup>. Notably, treatment of mice with the DC mitogen, Flt3L, has a hepatoprotective phenotype in AILI<sup>69</sup>. However, the lack of markers which are completely exclusive to DCs has been the main limitation in this area. Standardly used makers such as CD11c and MHC II are also expressed on macrophage subsets, making it difficult to interpret results of depletion studies using the CD11c-diphtheria toxin system<sup>70</sup>. Hence, it currently remains challenging to draw specific conclusions regarding DC function in the context liver regeneration.

**Figure 1 Immune regulation in liver homeostasis.**



**Figure 1.** In steady state, most of the hepatic parenchyma is comprised of quiescent hepatocytes. The NPCs reside in the sinusoids, except for HSCs, which are located in the space of Disse. The APCs (KCs, LSECs & DCs) flank the sinusoids, removing antigens from circulation via phagocytosis. KCs line the sinusoids to survey the circulation for pathogens, antigens and apoptotic bodies. Pathogen interactions can activate other innate immune cells such as NK cells via cytokine release, which can aid in the killing of pathogens or infected cells through TRAIL/IFN- $\gamma$  mediated mechanisms. LSECs interact with T cells to induce tolerance, generating tolerant T cells and initiating apoptosis of activated T cells (CTLs). Immunosuppressive mediators (IL-10, TGF $\beta$  and PGE2) released by APCs can also promote tolerance in the liver. Abbreviations: CTL, cytotoxic T cells; DC, dendritic cells; HSC, hepatic stellate cells; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; KC, Kupffer cells; LSEC, liver sinusoidal endothelial cells; NK, natural killer cells; PGE2, prostaglandin E2; TGF- $\beta$ , transforming growth factor- $\beta$ ; TRAIL, TNF-related apoptosis-inducing ligand.

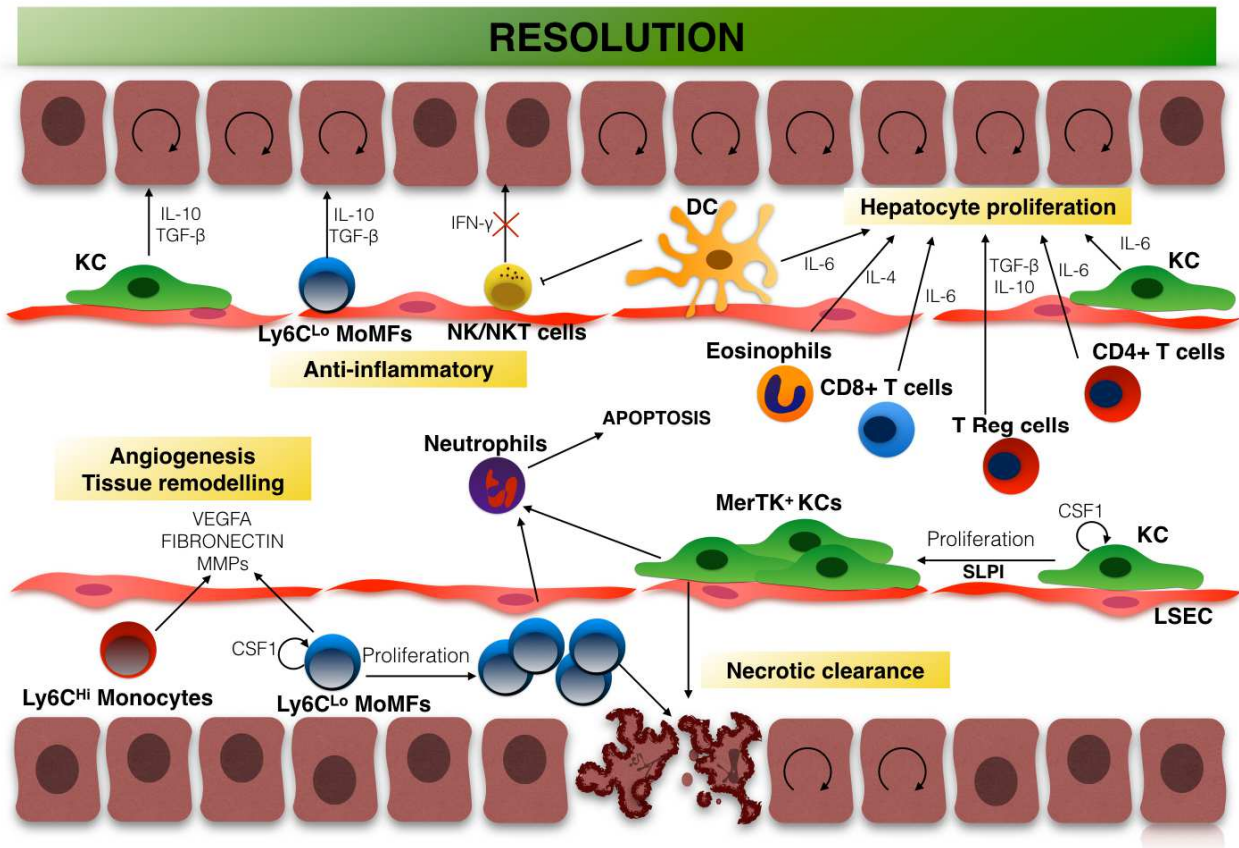


303 **Figure 2A Early stages of acute liver injury (necroinflammatory phase).**

**Figure 2A.** In response to hepatocyte death various DAMPs such as HMGB1 and H<sub>2</sub>O<sub>2</sub> are released and inflammation is initiated. DAMPs activate KCs and neutrophils via TLR-interaction and recruit them to the site of injury. KCs and neutrophils can initiate clearance of the necrotic debris, and promote hepatic injury and cell death via release of reactive oxygen species (ROS), NO, IL-1 $\beta$ , IL-6 and TNF- $\alpha$ . Chemoattractants such as CCL2, and IL-17 released by KCs, DCs and T cells facilitate the infiltration of bone marrow-derived leukocytes from the systemic circulation to the necrotic sites. During the early stages Ly6C<sup>Hi</sup> monocytes proliferate and expand via CSF-1, and contribute to inflammation via release of pro-inflammatory mediators (IL-1 $\beta$ , TNF- $\alpha$ ). NK and NKT cells also contribute to this pro-inflammatory phenotype through IFN- $\gamma$ -dependent mechanisms. Ly6C<sup>Hi</sup> monocytes can differentiate into a transient population of Ly6C<sup>Lo</sup> MoMFs, which are phenotypically distinct from KCs. Abbreviations: CCL2, C-C motif chemokine ligand-2; CXCL, C-X-C motif ligand, CSF1, Colony stimulating factor; DAMPs, damage associated molecular patterns; HMGB1, heat mobility group box protein-1; Hsp70, Heat shock protein-70; MoMFs, monocyte derived macrophages; NKT cells, Natural killer cells; NO, nitric oxide; ROS, reactive oxygen species; TNF- $\alpha$ , Tumour necrosis factor- $\alpha$ .



304 **Figure 2B Immune cell contribution to resolution following acute liver injury.**



**Figure 2B.** KC numbers are re-established via self-renewal and reprogramming of KCs and Ly6C<sup>Lo</sup> MoMFs to a pro-resolution macrophage phenotype promotes neutrophil apoptosis. Ly6C<sup>Lo</sup> MoMFs and KCs display enhanced phagocytic capabilities and cellular debris is cleared via these cells. The lost hepatic parenchyma is replaced via hepatocyte proliferation, induced by mitogens such as HGF, IL-6, IL-4, or TGF-β released by immune cells. Inflammation is dampened, with reduction of pro-inflammatory cells (Ly6C<sup>Hi</sup> monocytes), inhibition of IFN-γ release by NK/NKT cells and release of anti-inflammatory mediators. Ly6C<sup>Hi</sup> monocytes reprogram to a pro-resolution phenotype, and together with Ly6C<sup>Lo</sup> MoMFs they promote angiogenesis and tissue remodeling. Abbreviations: HGF, Hepatocyte growth factor; MMPs, matrix metalloproteinases; SLPI, Secretory leukocyte protease inhibitor; VEGFA, vascular endothelial growth factor A.

## Neutrophils

Neutrophils are the most abundant innate immune cells in the systemic circulation, and are very early responders to infection and inflammation. In the steady state liver, there are relatively low numbers of neutrophils<sup>71</sup>. Inappropriate activation of neutrophils can contribute to persistent inflammation and promote severe liver damage<sup>41,72</sup>. Neutrophil-mediated liver injury is reported in models of hepatic ischemia-reperfusion injury<sup>73</sup>, alcoholic hepatitis<sup>74</sup>, and endotoxemia<sup>75</sup>. However, their role in liver regeneration is still debated. There are limited studies looking at the role of neutrophils following PHx. One study reports that neutrophils promote liver regeneration through interaction with intracellular adhesion molecule (ICAM-1), leading to KC-dependent release of hepatocyte mitogens IL-6 and TNF- $\alpha$ <sup>34</sup>. Neutropenic mice show delayed liver regeneration and reduced hepatic levels of TNF- $\alpha$  and IL-6<sup>34</sup>. Furthermore, significant changes in neutrophil phenotype are observed in patients who have undergone PHx. This has been proposed to be important in defense against the influx of gut derived endotoxins following hepatic resection<sup>76,77</sup>.

In the context of ALI, there is a huge infiltration of neutrophils from the circulation into the site of injury during the early phase<sup>47</sup>. HMGB1 and lipid peroxidation products from dying hepatocytes and pro-inflammatory mediators such as TNF- $\alpha$  and IL-1 released from KCs, can activate neutrophils and mediate their recruitment into the hepatic sinusoids<sup>78–80</sup>. Neutrophil chemotaxis from sinusoids to injury sites is regulated by chemokine receptor 2 (CXCR2) and formyl peptide receptor 1 (FPR1)<sup>78,79,81</sup>, leading to neutrophil accumulation, “patrolling” and clearance of necrotic debris<sup>41,75</sup> (Figure 2A).

The studies interrogating the functional role of neutrophils in AILI have relied mainly on inducing neutropenia using anti-GR-1 antibodies. Depletion of neutrophils in mice prior to APAP overdose, decreased neutrophil recruitment and protected against liver injury<sup>82</sup>. Conversely, anti-GR-1-mediated neutrophil depletion induced after APAP overdose did not protect against liver injury<sup>72</sup>. The protective phenotype seen with pretreatment of anti-Gr-1 was attributed to a preconditioning effect, where hepatoprotective genes are induced by KCs activated via antibody-tagged neutrophils in the sinusoids, prior to APAP challenge<sup>72</sup>. Similar attenuation of hepatic injury is also seen when neutrophil infiltration to the liver parenchyma is prevented by CXCR2-FPR1 antagonism<sup>79,83</sup>. This neutrophil-induced cytotoxicity in AILI has been attributed to high expression of inducible nitric oxide synthase (iNOS), which produces NO, a potent mediator of tissue damage<sup>83</sup> (Figure 2A). More recent studies show that neutrophil-sensing of intracellular DNA deposits at necrotic sites are a pathogenic feature of AILI, which activates the TLR9/NF- $\kappa$ B and IL-33/ST2 pathways<sup>84</sup>. Blocking these interactions resulted in a significant reduction in systemic inflammation, liver neutrophil recruitment and hepatotoxicity<sup>41,84</sup>.

Conversely, inhibiting neutrophil interaction with target cells, or preventing neutrophil-derived oxidant stress show no effect on hepatotoxicity<sup>71,85,86</sup>. The interplay between neutrophils and other immune cells may also have functional relevance. Infiltrating monocytes, MDMs and neutrophils were shown to coincide spatially and temporally during the necroinflammatory and resolution phases of AILI<sup>47</sup>. During the necroinflammatory phase, Ly6C<sup>Hi</sup> monocytes promote neutrophil activity which facilitates clearance of necrotic debris, whilst during the resolution phase Ly6C<sup>Lo</sup> MoMFs and KCs facilitate neutrophil clearance<sup>47</sup> (Figure 2B). It is

important to note that the studies in this area to date have not addressed whether neutrophils can influence hepatocyte proliferation in the context of liver regeneration.

Clinical assessment of explant livers and blood from patients who developed ALF from AILI show significant neutrophil dysfunction, including impaired bactericidal function, akin to that seen in severe sepsis<sup>87</sup>. Neutrophilic indices can be important clinical biomarkers of AILI, and indicators of the development of organ dysfunction and increased susceptibility to sepsis<sup>87</sup>. There is evidence that activated and functioning neutrophils persist in the liver during the later phase of AILI, suggesting a potential pro-reparative role for neutrophils<sup>88</sup>. Whether neutrophils have a beneficial or deleterious effect in AILI remains controversial, with variable findings in both mouse and human studies<sup>87,88</sup>. The contradictory evidence in mice may reflect differences in experimental approaches and techniques, such as animal strains, APAP dosage and mode of administration, and neutrophil depletion strategies. Neutrophils may play different roles in AILI at different times during the disease process, and understanding the precise signals that govern a potential switch between pro-injury and pro-reparative neutrophil phenotypes may identify novel therapeutic targets. Furthermore, interactions between neutrophils and other leukocytes (infiltrating and resident) during AILI warrants further study and as this may also provide more insight into their role in AILI.

## **Eosinophils**

Eosinophils have been extensively studied in the context of type 2 innate immunity, mainly in parasitic infections. Their role in liver regeneration and repair was largely unexplored until recently. A direct role for eosinophils in liver regeneration was

demonstrated by Goh et al (2013), where eosinophil-derived IL-4 signals were shown to promote hepatocyte proliferation following PHx and acute carbon tetrachloride (CCL4) liver injury. Mice lacking eosinophils show impaired hepatocyte proliferative capacity following PHx and CCL4 induced liver injury. Increased eosinophil recruitment to the liver following injury was accompanied by elevated expression of eotaxin-1, a potent eosinophil chemoattractant<sup>89,90</sup>. A similar eosinophil-mediated type 2 cytokine response is seen in muscle injury, where this pathway regulates muscle regeneration via IL-4/IL-4Ra signaling<sup>91</sup>. Together, these data suggest an important regulatory role of eosinophils in tissue regeneration.

Although there are no animal studies investigating the role of eosinophils in ALI, immunohistochemical analysis of livers from patients following drug-induced liver injury, including some patients with APAP overdose, show significant eosinophil infiltration<sup>92</sup>. Similar to the observation in mice<sup>89</sup>, this appears to be related to eotaxin expression<sup>92</sup>. Additionally, eosinophilia was found to be associated with a better prognosis in patients with acute liver injury secondary to drug-related aetiologies<sup>93</sup>.

## **Lymphocytes**

### **Innate Lymphocytes**

Lymphocytes account for approximately 25% of hepatic NPCs, and innate lymphocytes constitute approximately 50% of total hepatic lymphocytes in humans<sup>26,70</sup>. They are comprised of natural killer (NK) cells, natural killer T (NKT) cells and innate lymphoid cells (ILCs)<sup>70</sup>. NK cells are a major component of the innate

immune response, they recognize and destroy foreign pathogens, release cytokines/chemokines and mediate cytotoxic lysis of virus infected hepatocytes and tumour cells<sup>94</sup> (Figure 1). NKT cells on the other hand cells are a heterogeneous subset of lymphocytes expressing both NK and T cell surface markers<sup>95</sup>. ILCs are a recently identified heterogeneous group of cells which share similarities to NK cells. However, to their recent discovery we know very little about these cells in the context of liver regeneration<sup>96</sup>.

Both NK and NKT cells are activated following hepatic injury, displaying increased levels of cytotoxicity<sup>97,98</sup>. However, whether these cells drive injury or regeneration and repair is still unclear. As seen with macrophages and DCs, there is an overlap in cell surface markers between NK and NKT cells. This means studying the differential roles of these cells during liver injury is challenging. Following PHx, there is an accumulation of NK and NKT cells in the liver<sup>98,99</sup>. Preferential depletion of NK cells via anti-ASGM-1 treatment suggests they are negative regulators of liver regeneration, partly through IFN- $\gamma$  dependent mechanisms<sup>98</sup>. In their absence hepatocyte proliferation is enhanced with no apparent changes in liver injury or rate and amount of liver mass restoration<sup>98</sup>. However, it should be noted that the anti-ASGM-1 depletion strategy used in this study also has off-target effects on basophils<sup>98,100</sup>. Simultaneous depletion of both NK and NKT cells inhibits the regenerative response after PHx, due to a reduction in the pro-regenerative cytokines TNF- $\alpha$ , IL-6, HGF and IL-4<sup>99</sup>. The authors suggest that cross talk occurs between NK/NKT cells and KCs and together this results in the production of pro-regenerative cytokines<sup>99</sup>. Spatial correlation of KCs and NK cells in the sinusoids after PHx

supports this theory<sup>97</sup>. Recent findings implicate ILCs and NK cells as the main producers of IL-22 post-PHx which is required for efficient liver regeneration<sup>101</sup>.

The contribution of NK/NKT cells to ALI is not fully elucidated. Early reports of a positive correlation between activation of NK/NKT cells and pathogenesis of ALI has been found linked to dimethyl sulfoxide (DMSO), a solvent used to dissolve APAP<sup>102,103</sup>. DMSO can influence NK/NKT cell response and manipulating NK/NKT cells in the absence of DMSO failed to display any hepatoprotective effects<sup>103</sup>.

NK/NKT cells have been indirectly implicated in the pathogenesis of ALI. IFN- $\gamma$ , mainly released by NK cells, has been implicated as a mediator of tissue injury and inflammation following ALI<sup>68</sup>. Whereas, NKT cell deficient mice are more sensitive to APAP, as they show increased expression and activity of cytochrome P450 2E1 (CYP2E1); an enzyme required for APAP metabolism<sup>42</sup>. However, CYP2E1 expression is only increased under starvation<sup>42</sup>. So far there is no evidence to suggest either a direct protective or detrimental role of NK/NKT cells in the pathogenesis of ALI. ILCs have been shown to have both protective and pathogenic roles in liver diseases such as viral hepatitis and fibrosis, however their specific role in ALI has not been investigated<sup>104</sup>. As there are common cell surface markers between NK cells and ILCs. previous studies which define murine NK cells on the basis of NK1.1 and CD3 need to be re-evaluated<sup>102,103</sup>.

### **Adaptive lymphocytes**

T cells and B cells are major players in adaptive immunity, for example mediating cellular and humoral responses against pathogens and cancer cells<sup>26</sup>. There are a



significant number of hepatic T cells, localized to the portal tracts and parenchyma in a healthy liver, which are phenotypically different from circulating lymphocytes<sup>105</sup>. T cells are involved in maintaining tolerance in the liver, however they are also major players in hepatic injury and inflammation<sup>106</sup>. The T cell compartment is comprised of highly heterogeneous cells and like macrophages, these cells are capable of mediating both pro-inflammatory or anti-inflammatory responses<sup>70</sup>. CD4<sup>+</sup> T cells (T helper or T<sub>H</sub> cells) and CD8<sup>+</sup> T cells (cytotoxic T cells) are the two broad subtypes<sup>106</sup>. Depending on transcriptional profiles and cytokine secretion, T<sub>H</sub> cells has been further divided into four main subsets (T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17 and regulatory T<sub>REG</sub> cells)<sup>70</sup>. In addition to classical T cells, there are also a small proportion of non-classical T cells in the liver, termed  $\gamma\delta$ -T cells<sup>70</sup>.

The main evidence suggesting a role for adaptive lymphocytes in liver regeneration has come from experiments on RAG1<sup>-/-</sup> (T and B cell deficient) mice. Following PHx RAG1<sup>-/-</sup> mice show increased mortality and extensive hepatic injury, indicating that adaptive immune cells are critical in regulating liver regeneration<sup>107</sup>. Using hepatocyte proliferation, measurement of necrosis and mortality as readouts, T cell specific knockout experiments demonstrate that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, but not  $\gamma\delta$  T cells are required for normal liver regeneration<sup>107</sup>. This regulation occurs through a T cell-derived lymphotoxin (LT) axis, whereby LT expression by T cells promotes liver regeneration through direct hepatocyte contact and via the IL-6 pathway<sup>107</sup>. In addition to T cells, B cells also express LT, but B cell derived LT has not been shown to be linked to liver regeneration<sup>107</sup>.



The role of T cells in ALI has not been investigated in depth, but current research suggests that CD4<sup>+</sup> T (T<sub>H</sub>) cells play a pivotal role while CD8<sup>+</sup> T cells do not appear to modulate ALI<sup>108</sup>. Experiments using different strains of mice have shown that the T<sub>H</sub>1/T<sub>H</sub>2 balance is important in ALI; as T<sub>H</sub>1 dominant mice (C57BL/6) are more susceptible to ALI than T<sub>H</sub>2 dominant mice (BALB/c)<sup>109</sup>. This difference was attributed to cytokine release, rather than differences in APAP metabolism. Expression of TNF- $\alpha$  and IFN- $\gamma$ , was elevated in T<sub>H</sub>1 dominant mice<sup>68,110</sup> while T<sub>H</sub>2 dominant mice had high expression of the hepatoprotective cytokine IL-6<sup>107,109</sup>. There is also an increase in T<sub>REG</sub> cells in the liver post-ALI, and they display a protective phenotype, by suppressing inflammation and releasing hepatocyte mitogens such as IL-10 and TGF- $\beta$  (Wang et al., 2015). Currently, we do not know the signals driving T<sub>REG</sub> accumulation in the liver following ALI. There is likely a complex interplay between T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>REG</sub> subsets which may regulate both the progression and resolution of hepatic injury<sup>108,109</sup>.

Recently T<sub>H</sub>17 cells have been implicated in the immune response during the initial stages of ALI<sup>111</sup>. T<sub>H</sub>17 cells increase significantly following APAP overdose and they have been identified as the main cell type to promote IL-17 signaling, which is associated with the release of pro-inflammatory and neutrophil-mobilizing cytokines<sup>111,112</sup>. Furthermore, patients with ALI have increased levels of IL-17 and IL-21 (another T<sub>H</sub>17-secreted cytokine) in their serum<sup>113</sup>. IL-17 can be produced by many cell types, including NK cells and  $\gamma\delta$  T cells<sup>113</sup>. During ALI,  $\gamma\delta$  T cells can have been shown to secrete IL-17, as a response to HMGB1-mediated release of IL-23 by hepatic macrophages<sup>114</sup>. This interaction between macrophages and  $\gamma\delta$  T cells results in neutrophil recruitment into the injury sites, promoting inflammation<sup>114</sup>.

(Figure 2). Additionally, treatment with baicalin, a flavonoid compound has been found to elicit hepatoprotective effects in ALI, by attenuation of  $\gamma\delta$  T cells and IL-17 expression<sup>115</sup>.

## Conclusion

It is clear that immune cells are key regulators of liver regeneration and repair. However, the precise role of specific immune cell subpopulations is highly context-dependent, with both innate and adaptive immune cells playing very different roles at different stages of the disease process. Importantly, a broad categorization of cells as negative and positive regulators of liver regeneration is not sufficient, as many immune cells types are significantly more heterogeneous in terms of function than we previously appreciated. Further work to dissect the hepatic immune cell landscape at high resolution will greatly facilitate the identification and characterization of the key pro-regenerative immune cell subsets during liver injury and repair. Such studies will be essential to better define the immune components of the regenerative niche in the liver, which will guide the development of highly targeted therapeutic strategies to promote hepatic regeneration in patients with liver disease.

## Acknowledgements

The authors acknowledge the support of the Wellcome Trust and the Medical Research Council.

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